

## Promoter Hypermethylation of High-in-normal 1 Gene in Primary Nasopharyngeal Carcinoma<sup>1</sup>

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### ABSTRACT

**Purpose:** The methylation of high-in-normal-1 (HIN-1) gene promoter in undifferentiated nasopharyngeal carcinoma (NPC) is studied.

**Experimental design:** The methylation status of HIN-1 in NPC cell lines, primary NPC, paired nasopharyngeal swabs, paired throat-rinsing fluid, and paired peripheral blood was assessed by methylation-specific PCR assay. The relationship between HIN-1 promoter methylation and transcription in NPC cell lines was evaluated by reverse transcription-PCR and demethylation agent treatment (5-aza-2-deoxycytidine).

**Results:** Hypermethylated promoter was observed in five of five (100%) NPC cell lines and not found in three normal nasopharyngeal outgrowths, two tonsil epithelial cell cultures, and two skin fibroblast cultures. Reverse transcription-PCR assay indicated that HIN-1 transcription was significantly down-regulated in the NPC cell line with promoter methylation. Treatment with demethylation agent, 5-aza-2-deoxycytidine, restored HIN-1 transcription in the NPC cell line. Methylated HIN-1 promoter was found in 36 of 47 (77%) primary NPC tumors and not found in the normal nasopharyngeal biopsies. Methylated HIN-1 promoter was detected in 12 of 26 (46%) nasopharyngeal swabs, 5 of 26 (19%) throat-rinsing fluids, 2 of 11 (18%) plasmas, and 5 of 11 (46%) buffy coats of peripheral blood of the NPC patients but was not detectable in all normal controls.

**Conclusion:** HIN-1 promoter hypermethylation is common in NPC. Methylated promoter DNA in nasopharyngeal

swab, throat-rinsing fluid, and peripheral blood might be potentially useful as tumor marker for screening of NPC.

### INTRODUCTION

Aberrant methylation in 5' CpG island of promoter region interferes with transcription initiation by reducing binding of transcription factors (1) and recruiting transcription repressor that binds to the methylated DNA specifically (2). Promoter hypermethylation is observed in a variety of human cancers and responsible for epigenetic silencing of tumor suppressor genes (3-6). Gene methylation profile varies in different cancers (7). We have shown previously that methylation of E-cadherin gene of oral tongue cancer can cause transcriptional silencing and down-regulation of its protein expression, thereby inducing more metastatic potential and higher risk of recurrence after treatment (8). The study of patterns of gene silencing would therefore help to understand and predict the overall phenotype, proliferation, invasiveness, metastatic potential, and responsiveness to various treatments of cancers.

HIN-1<sup>3</sup> is a putative cytokine gene located at 5q35-tel (9). Expression of HIN-1 is significantly down-regulated in human breast cancer and its preinvasive lesions. The decrease in expression is accompanied with hypermethylation of the promoter region. Reintroduction of HIN-1 inhibits cell growth in breast cancer cell lines (9). The role of methylation of HIN-1 in NPC development has not been reported. In this study, we evaluated the role of HIN-1 methylation in the tumorigenesis of NPC. Because tumor suppressor gene promoter methylation is a potential tumor marker in various clinical samples, including saliva, sputum, bronchial brush, urine, plasma/serum, and peripheral blood cells (10-15), we also investigated detection rates of methylated HIN-1 promoter DNA isolated from plasma, peripheral blood cells, nasal swabs, and throat-rinsing fluid of NPC patients.

### MATERIALS AND METHODS

**Cell Cultures.** Five NPC cell lines (CNE-1, CNE-2, CNE-3, M-1, and SUNE-1), three adenoid-derived epithelial cell primary cultures, two tonsil-derived epithelial cell primary cultures, and two skin-derived fibroblast primary cultures were examined. All cell lines and skin-derived fibroblasts were maintained in RPMI (Life Technologies, Inc., Rockville, MD) supplemented with 10% FCS (Life Technologies, Inc.). The primary adenoid and tonsil epithelial cultures were maintained in Keratinocyte serum-free medium (Life Technologies, Inc.) together with the growth supplements supplied (Life Technologies, Inc.).

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<sup>3</sup> The abbreviations used are: HIN-1, high in normal-1; NPC, nasopharyngeal carcinoma; MSP, methylation-specific PCR; RT-PCR, reverse transcription-PCR.

**Patients and Samples.** Forty-seven primary NPC tumors were obtained with endoscopic biopsy. Twelve normal adenoid tissues obtained at adenoidectomy were included as normal control. Samples were stored at liquid nitrogen for transportation at  $-70^{\circ}\text{C}$  before DNA purification. All tumor samples were histopathologically confirmed as undifferentiated NPC according to the WHO classification.

Peripheral blood samples were collected from 11 NPC patients and 16 healthy volunteers. Whole blood was first centrifuged at  $400 \times g$  for 10 min at room temperature. The plasma and buffy coat layers were aliquoted and stored in a plain tube separately. An additional centrifugation at  $1000 \times g$  for 10 min was performed on the plasma fraction to remove cellular debris. Four-hundred  $\mu\text{l}$  of plasma and 200  $\mu\text{l}$  of peripheral blood cells were used for DNA extraction.

Nasopharyngeal swabs and throat-rinsing fluids were taken from 27 NPC patients. Fourteen normal nasopharyngeal swabs and 20 normal throat-rinsing fluids were collected from healthy volunteers as controls. The nasopharyngeal swabs were taken with the cotton tip of dress applicators through the nose before nasoendoscopy. The cells collected at the cotton tip were dipped into a 2-ml bottle of saline with gentle shaking. The throat-rinsing fluid was collected by rinsing the mouth and throat with 20 ml of normal saline. The cells of the throat-rinsing fluid were collected by centrifugation. All of the cell samples were stored at  $-70^{\circ}\text{C}$  until use.

**MSP and RT-PCR.** The MSP was performed as described by Herman *et al.* (16, 17) and in our previous publication. DNA bisulfite modification was carried out by the CpGenome DNA Modification Kit (Intergen, New York, NY). Unmethylated cytosine would be deaminated by sodium bisulfite and converted into uracil. In contrast, methylated cytosine would remain unchanged. After bisulfite modification, the gene sequences of methylated and unmethylated HIN-1 were different and therefore could be distinguished by using different primer pairs in separate PCR reaction. One  $\mu\text{g}$  of the purified DNA was subjected to bisulfite modification.

The modified DNA was then eluted in 50  $\mu\text{l}$  of  $\text{H}_2\text{O}$ . One-fifth (200 ng) of the modified DNA was analyzed by MSP. With the conversion factor of 6.6 pg per diploid cell, the amount of DNA is equivalent to  $3 \times 10^4$  cells in the PCR reaction. RNA isolation, primer sequences, and reaction parameters for the MSP and RT-PCR were performed as described by Krop *et al.* (9).

**Demethylation by 5-aza-2'-deoxycytidine.** NPC cell lines were grown in medium containing 5-aza-2'-deoxycytidine (range: 0–10  $\mu\text{M}$ ) for 4 days. Fresh medium without demethylating agent was then replaced, and the cell lines were allowed to grow for 24 h before analysis.

## RESULTS

**Promoter Methylation of HIN-1 in NPC Cell Lines and Normal Primary Cultures.** Methylated HIN-1 was observed in all five (100%) NPC cell lines. CNE-1, CNE-2, and SUNE-1 contained both unmethylated and methylated promoters, whereas CNE-3 and M1 had only methylated promoter (Fig. 1a).

Promoter methylation of HIN-1 was not observed in any of the three adenoid-derived epithelial cell cultures, two tonsil-

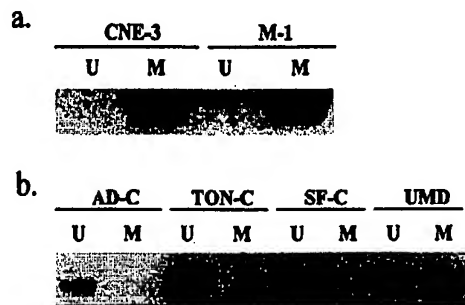


Fig. 1 a, methylation analysis of HIN-1 promoter in NPC cell lines; b, methylation analysis of the normal cultures with universal methylated DNA as positive control. U, reaction specific for unmethylated HIN-1 promoter; M, reaction specific for methylated HIN-1 promoter; AD-C, adenoid-derived epithelial cell cultures; TON-C, tonsil-derived epithelial cell cultures; SF-C, skin-derived fibroblast cultures; UMD, universal methylated DNA.

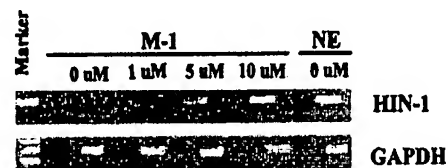


Fig. 2 HIN-1 expression in NPC cell lines, normal adenoid-derived epithelial cell cultures, and the effect of 5-aza-2'-deoxycytidine on HIN-1 expression in NPC cell lines. RT-PCR was used to evaluate HIN-1 expression. Glyceraldehyde-3-phosphate dehydrogenase expression was used as the internal reference gene.

derived epithelial cell cultures, and two skin-derived fibroblasts (Fig. 1b).

**Demethylation by 5-aza-2'-deoxycytidine and RT-PCR.** To evaluate whether methylation of HIN-1 promoter is associated with transcription silencing of HIN-1 in the NPC cell lines, RT-PCR and demethylation treatment were performed on the NPC cell line, M1, which has purely methylated HIN-1 promoter. Treatment of M1 with 5-aza-2'-deoxycytidine restored transcription of HIN-1 mRNA (Fig. 2).

**Hypermethylation of HIN-1 Promoter in NPC and Normal Nasopharyngeal Tissue.** To evaluate the prevalence of HIN-1 methylation in primary NPC, we examined the methylation status of HIN-1 in 47 primary NPC. Fig. 3 illustrates the representative MSP results. Methylated HIN-1 promoter was found in 36 of 47 (77%) primary NPC. Methylated HIN-1 was not detected in all of the 12 normal adenoid tissues. Table 1 summarizes the clinicopathological characteristics of the NPC patients and HIN-1 methylation.

**Detection of Hypermethylation of HIN-1 Promoter in Nasopharyngeal Swab and Throat-rinsing Fluid of NPC Patient.** To evaluate the potential diagnostic value of HIN-1 methylation in NPC, we evaluated the detection rate of HIN-1 methylation in 26 paired nasopharyngeal swabs and throat-rinsing fluid of the NPC patients. Methylated HIN-1 promoter was found in 12 of 26 (46%) nasopharyngeal swabs and 5 of 26

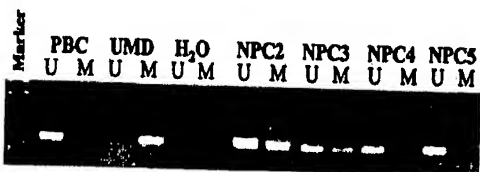


Fig. 3 Representative MSP analysis of primary NPC tissues. U, reaction specific for unmethylated HIN-1 promoter; M, reaction specific for methylated HIN-1 promoter; UMD, universal methylated DNA; PBC, DNA extracted from peripheral blood cells obtained from a normal individual; NPC2-5, primary NPC cases 2-5. Marker, 100-bp DNA ladder.

Table 1 Summary of clinical/pathological characteristics of the NPC patients and HIN-1 methylation

NPC samples	No.	HIN-1 methylation
Sex		
Male	37	29 (78%)
Female	10	7 (70%)
AJCC <sup>a</sup> staging		
I	2	2 (100%)
II	15	10 (67%)
III	15	14 (93%)
IV	15	11 (73%)

<sup>a</sup> AJCC, American Joint Committee on Cancer.

(19%) throat-rinsing fluid. Methylated HIN-1 was not found in the nasopharyngeal swabs and throat-rinsing fluid of all healthy individuals. Tables 2 and 3 summarize the detection rate of hypermethylated HIN-1 promoter in the nasopharyngeal swab and throat-rinsing fluid of the 26 patients.

**Detection of Hypermethylated HIN-1 Promoter in Peripheral Blood of NPC Patient.** To evaluate the potential diagnostic value of hypermethylated HIN-1 as serological tumor marker in the screening of NPC, MSP was performed on DNA extracted from plasma and buffy coat layer of 11 NPC patients. Methylation of HIN-1 promoter was detected in 2 of 11 (18%) paired plasmas and 5 of 11 (46%) peripheral blood cells. The overall detection sensitivity was 6 of 11 (55%) in blood. Methylated HIN-1 was not found in all of the 16 normal control peripheral blood. Table 4 summarizes the methylated HIN-1 promoter in primary tumors and paired peripheral blood.

## DISCUSSION

Aberrant methylation of tumor suppressor genes is common in NPC (17-20). In this study, we reported another candidate tumor suppressor gene, HIN-1, which is also highly methylated in undifferentiated NPC of Hong Kong Chinese. HIN-1 is a putative cytokine that was initially identified by using serial analysis of gene expression. HIN-1 is highly expressed in the normal mammary ductal epithelial cells and down-regulated in breast cancer cell lines and breast cancers (9). The reduction in gene expression in cancer cell lines and cancer tissues is associated with promoter hypermethylation (9). Down-regulation of HIN-1 mRNA and protein by promoter hypermethylation has been confirmed by Northern blotting, MSP, and Western blotting (9). *In vitro* expression of HIN-1 protein in cancer cell lines

Table 2 Summary of HIN-1 methylation in primary tumor, nasopharyngeal swab, and throat-rinsing fluid<sup>a</sup>

Case	AJCC <sup>a</sup> staging	Sex	Age	Primary tumor	Nasopharyngeal swabs	Throat-rinsing fluid
NPC-1	III	M	47	+	0	0
NPC-2	II	M	54	+	0	0
NPC-3	IV	M	53	+	+	+
NPC-4	IV	M	64	+	+	0
NPC-5	II	F	30	0	0	0
NPC-6	IV	M	46	+	+	+
NPC-7	IV	M	51	+	+	0
NPC-8	IV	M	59	+	+	+
NPC-9	II	F	61	+	+	0
NPC-10	II	M	53	+	0	0
NPC-11	IV	M	46	+	0	0
NPC-12	IV	M	46	0	0	0
NPC-13	II	M	54	+	+	0
NPC-14	I	F	41	+	+	0
NPC-15	III	F	46	0	0	0
NPC-16	II	M	42	+	0	0
NPC-17	II	M	42	+	0	0
NPC-18	I	F	60	+	+	0
NPC-19	IV	M	57	+	+	+
NPC-20	III	M	48	+	+	0
NPC-21	IV	M	30	0	0	0
NPC-22	IV	M	49	0	0	0
NPC-23	II	F	48	0	0	0
NPC-24	II	M	40	0	0	0
NPC-25	III	M	62	+	+	+
NPC-26	II	M	55	0	0	0

<sup>a</sup> +, HIN-1 methylation; 0, unmethylated HIN-1.

<sup>b</sup> AJCC, American Joint Committee on Cancer.

Table 3 Detection rate of HIN-1 methylation in nasal swabs and throat-rinsing fluids of NPC patients<sup>a</sup>

	MSP+ (%)	MSP- (%)
A. Nasopharyngeal swab		
NPC (n = 26)	12 (46%)	14 (54%)
Normal (n = 14)	0 (0%)	14 (100%)
	Sensitivity = 46%	Specificity = 100%
B. Throat-rinsing fluid		
NPC (n = 26)	5 (19%)	21 (81%)
Normal (n = 20)	0 (0%)	20 (100%)
	Sensitivity = 19%	Specificity = 100%

<sup>a</sup> MSP+, HIN-1 methylated; MSP-, HIN-1 unmethylated.

would negatively regulate colony formation. HIN-1 also plays a role in regulation of epithelial cell proliferation, differentiation, or morphogenesis (9).

Methylated HIN-1 promoter was found in 77% NPC and all NPC cell lines. It implied that transcriptional silencing of HIN-1 pathway might be involved in NPC tumorigenesis. High frequency of methylation was also present in early stages I and II NPC. It demonstrated that this might be an early event.

We have demonstrated previously that methylated promoter DNA might be used as a serological tumor marker provided that the selected gene promoter is frequently methylated in the primary tumors but not in normal controls (17). The high incidence of HIN-1 methylation in the NPC cell lines and tumor tissues but not in normal controls prompted us to further eval-

Table 4 Summary of HIN-1 methylation in primary tumors, plasma, and buffy coat<sup>a</sup>

Case	AJCC <sup>b</sup> staging	Age	Sex	Primary tumor	Plasma	Buffy coat
NPC-27	III	34	male	+	0	0
NPC-28	II	38	male	+	0	0
NPC-29	III	56	male	+	0	0
NPC-30	III	34	female	+	0	0
NPC-31	III	45	male	+	+	0
NPC-32	III	45	male	+	0	+
NPC-33	II	45	male	+	0	+
NPC-34	III	34	male	+	0	+
NPC-35	III	46	male	+	0	0
NPC-36	IV	74	male	+	0	+
NPC-37	II	42	male	+	+	+

<sup>a</sup> +, HIN-1 methylation; 0, unmethylated HIN-1.

<sup>b</sup> AJCC, American Joint Committee on Cancer.

uate its clinical diagnostic value in peripheral blood, nasopharyngeal swab, and throat-rinsing fluid. The sensitivity of peripheral blood is 55% (18% plasma and 46% buffy coat) and 100% of prediction rate, with all serological methylation-positive patients having NPC. In our previous serological study, the conventional serological EBV viral capsid antigen IgA antibody titer has 90% sensitivity but had very low prediction rate with only 5% of serological-positive patients having NPC (21). The plasma-methylated DNA is a reflection of aberrant DNA being disseminated from the primary or metastatic tumors in the body. The methylated promoter DNA in buffy coat indicates the presence of circulating cancer cells in the process of distant metastasis. The sensitivity of methylated DNA markers might be further improved by increasing the amount of plasma and buffy coat in the protocol or by using quantitative PCR. The other possibility is to use a panel of multiple markers. In our previous study, death-associated protein kinase is one of the markers that can be considered in the batch of NPC serological methylation markers with high sensitivity and specificity (17).

Nasopharyngeal swab and throat-rinsing fluid are also possible screening methods for NPC (22). They can be easily obtained noninvasively and repeatedly from patients. The sensitivity of methylated HIN-1 was 67% for nasopharyngeal swab and 28% for throat-rinsing fluid. The specificity was 100%. The nasopharyngeal swab was a more sensitive method compared with throat-rinsing fluid probably because of the higher content of cancer cells in the swabs. In our protocol, the nasopharyngeal swab was taken from both sides of nose without the knowledge of the site of nasopharyngeal tumor, at the time before nasendoscopy. The technique is simple and can be applicable to general practitioners without the necessity of specialist otorhinolaryngologist. Again, the limiting factor is the absence of HIN-1 methylation in some NPC. Unlike peripheral blood in which we might be able to increase the amount of DNA with larger volume of blood, multiple markers are necessary to increase the sensitivity for screening of NPC.

Hypermethylated DNA may serve as potential molecular tumor markers because of its high specificity to differentiate cancer from normal tissues. In this study, we demonstrated that hypermethylated HIN-1 has high specificity in discriminating NPC patients from normal individuals in tissues and body fluids.

The methylated HIN-1 promoter was absent in the normal samples, including normal cultures, peripheral blood, nasal swabs, and throat-rinsing fluid. Overall, HIN-1 methylation had moderate levels of sensitivity for distinguishing primary NPC from healthy individuals. In case of screening of high-risk population, a panel of markers would be necessary to increase the sensitivity. EBV serological screening tests usually have high sensitivity but low prediction rate (23–25). Methylation markers might be used in combination with the conventional EBV antibody markers to enhance the specificity in NPC screening. Methylated promoter DNA in body fluids might also be used in assisting detection of minimal residual cancer after treatment based on the methylation profile of individual cancer of the patient before treatment. In both screening and monitoring applications, more markers should be identified in the future to increase its diagnostic sensitivity and specificity in the clinical application of methylated promoter DNA.

In conclusion, promoter methylation of HIN-1 is common in NPC. Aberrant methylation of HIN-1 promoter was observed in NPC but not in normal nasopharyngeal tissues. The possible detection of methylated HIN-1 promoter DNA in nasopharyngeal swab, throat-rinsing fluid, and peripheral blood suggested that it might be one of the potential useful tumor markers in assisting clinical screening and detection of minimal residual tumor after treatment of NPC.

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